EXHIBIT E

# Excision of selectable marker genes from transgenic plants

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Selectable marker genes are required to ensure the efficient genetic modification of crops. Economic incentives and safety concerns have prompted the development of several strategies (site-specific recombination, homologous recombination, transposition, and co-transformation) to eliminate these genes from the genome after they have fulfilled their purpose. Recently, chemically inducible site-specific recombinase systems have emerged as valuable tools for efficiently regulating the excision of transgenes when their expression is no longer required. The implementation of these strategies in crops and their further improvement will help to expedite widespread public acceptance of agricultural biotechnology



The genetic modification of plants potentially offers substantial improvements to agricultural practices, food quality, and human health. The success of these endeavors is determined both by the ability to deliver foreign DNA into the host plant and by the efficiency with which shoots or somatic embryos can be regenerated from transformed cells. Currently, the poor transformation efficiency of most crops necessitates the use of selectable marker genes to identify transgenic plants (Table 1). These conditional dominant genes either confer resistance to an antibiotic or herbicide that kills non-transformed cells or confer a metabolic advantage on transformed cells in the presence of a nontoxic selective agent. Genes that permit identification of transgenic plants in the absence of a selective agent, including regeneration-enhancing genes such as *ipt*[1,2], are defined here as screenable markers. The use of such genes in "marker-free" transformation is currently a feasible alternative to marker-based selection for species that can be regenerated by organogenesis or somatic embryogenesis[3]. As the continued expression of selectable markers in field grown plants is usually unnecessary, and may even be highly undesirable, several strategies have been developed to remove selectable markers after they have served their function.

Several other reviews on marker removal have been published recently[4–6]. Here we place particular emphasis on a most recent development—the use of inducible site-specific recombinase systems for efficient transgene deletion strategies—and suggest possible approaches toward their improvement.

## The rationale for marker elimination

The success of agricultural biotechnology, like that of any market-driven enterprise, relies ultimately on both consumer demand and the rate at which new products can be released commercially. The inclusion of marker genes in genetically modified (GM) crops has caused public concerns about the medical implications of consuming GM food and the environmental implications of growing GM crops (see Dale p. 567). Although it remains highly contentious that such concerns are warranted, the extreme difficulty of proving that selectable markers are indeed harmless has prevented unanimous public acceptance of plant biotechnology. Besides minimizing public concerns, strategies to eliminate marker genes also reduce the need for time-consuming and expensive safety evaluations. This reduces the cost of developing and marketing new GM products and expedites the commercial release of new products. In the foreseeable future, it is likely that regulatory legislation will strongly favor the absence of superfluous transgenic material in GM crops.

The majority of nuclear-encoded markers have no impact on plant growth or development in the absence of the selection agent. Usually, their continued presence neither compromises plant productivity nor is of any agronomic value (except with respect to herbicide resistance). However, the expression in a plastid of tens of thousands of copies of a marker gene in each cell of a transplastomic line[7] may be less innocuous. In cases where the marker protein may constitute as much as 10% of total soluble cellular protein[8], this is likely to incur a substantial metabolic burden. The absence of alternative technologies to ensure homoplasmic transformation without the use of selectable markers further underscores the importance of adopting effective strategies to eliminate chloroplast selectable markers once they have served their function in identifying transplastomic lines.

The advent of functional genomics and genome-wide expression profiling provides additional motivation for the development of rapid and efficient transgene removal mechanisms[9]. These technologies promise to identify the multigenic basis of many agriculturally important traits. As current transformation protocols severely limit the number of genes that can be introduced simultaneously, retransformation of a single line is the only rational approach toward selectively introducing multiple genes for complex traits such as productivity or stress tolerance. Co-incorporation of a different marker with each gene or set of genes is incompatible with safety concerns, expensive, and time consuming. Tissue culture regimes for transformant selection would have to be optimized for different species or varieties and the possible food safety and environmental impacts of different markers would have to be assessed on a case-by-case basis. There are also a limited number of constitutive promoters commonly used to express marker genes, and the repeated introduction of these promoters could conceivably activate gene-silencing mechanisms with adverse effects on the expression of novel genes of interest. Gene excision mechanisms permit the recycling of a single marker by its removal after each transformation step.

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**Table 1. Selectable markers currently used in transgenic plants confer resistance to antibiotics, herbicides or metabolic inhibitors**

| Gene | Gene product | Selection agent(s) | Gene source | Ref. |
|---|---|---|---|---|
| *nptII/neo*[P] | Neomycin phosphotransferase II | Kanamycin, neomycin, geneticin (G418), paromomycin, amikacin | *Escherichia coli*, transposon Tn*5* | 45 |
| *bar*[P] | Phosphinothricin acetyltransferase | Glufosinate, L-phosphinothricin, bialaphos | *Streptomyces hygroscopicus* | 45 |
| *pat*[P] | Phosphinothricin acetyltransferase | Glufosinate, L-phosphinothricin, bialaphos | *Streptomyces viridochromogenes* | 29,45 |
| *bla* | β-Lactamase | Penicillin, ampicillin | *Escherichia coli* | 45 |
| *aadA*[P] | Aminoglycoside-3′-adenyltransferase | Streptomycin, spectinomycin | *Shigella flexneri* | 45 |
| *hpt* | Hygromycin phosphotransferase | Hygromycin B | *Escherichia coli* | 45 |
| *nptIII* | Neomycin phosphotrasferase III | Amikacin, kanamycin, neomycin, geneticin (G418), paromomycin | *Streptococcus faecalis R* plasmid | 45 |
| *epsps/aroA*[P] | 5-Enoylpyruvate shikimate-3-phosphate | Glyphosate | *Agrobacterium CP4, Zea mays, Petunia hybrida* | 45 |
| *gox* | Glyphosate oxidoreductase | Glyphosate | *Achromobacter LBAA* | 45 |
| *bxn* | Bromoxynil nitrilase | Bromoxynil | *Klebsiella pneumoniae* var. *iozaenae* | 45 |
| *als* | Acetolactate synthase | Sulfonylureas, imidazolinones, thiazolopyrimidines | *Arabidopsis thaliana, Nicotiana tabacum, Brassica napus* | 45 |
| *cat*[P] | Chloramphenicol acetyltransferase | Chloramphenicol | Bacteriophage P1 *Cm*[R] | 7 |
| *TDC* | Tryptophan decarboxylase | 4-Methyltryptophan | *Catharanthus roseus* | 46 |
| *uidA/GUS*[P] | β-Glucuronidase | Cytokinin glucuronides | *Escherichia coli* | 47 |
| *xylA* | Xylulose isomerase | D-Xylose | *Thermoanaerobacterium thermosulfurogenes* | 48 |
| *manA* | Phosphomannose isomerase | Mannose-6-phosphate | *Escherichia coli* | 49 |
| *BADH*[P] | Betaine aldehyde dehydrogenase | Betaine aldehyde | *Spinacia oleracea* | 7 |

[P]Shown to be functional in plastids[6].

Finally, the development of effective strategies to precisely excise genetic elements might also find other valuable biotechnological applications. For example, inducible excision of an intervening DNA fragment between a promoter and a transgene could be used to regenerate lines expressing transgenes that affect embryo, seed, or seedling viability[10]. Recombinase-mediated excision of an antisense copy of a gene required for pollen viability restored male fertility in *Arabidopsis thaliana*[11]. This may provide a valuable approach to the production of commercially important hybrid seed and plants. Conversely, separation of a pollen- or tapetum-specific promoter and a "suicide" transgene by an excisable element could prevent possible gene flow into nontransgenic plants by cross-pollination. In GM crops engineered for tolerance to predation that is limited to inedible organs, it may be preferable to limit expression of anti-predation transgenes to organs not intended for human or animal consumption. Localized induction of a tightly regulated, chemically induced transgene excision mechanism might be preferable in instances where there are no stringently regulated tissue-specific promoters available for selective ablation of transgene expression in one plant organ but not in another[12].

## Sexual crosses or re-transformation of primary transformants

Irrespective of the application, any strategy for targeted gene elimination should aim to be both rapid and 100% efficient. Procedures that avoid an additional round of transformation or sexual crosses of the primary transformants are both quicker and less labor-intensive. Furthermore, cross-pollination is not compatible with breeding programs for crops that are vegetatively propagated or have long generation times, and repeated passage through tissue culture increases the incidence of somaclonal variation. Where gene excision strategies employ gene products that modify DNA, rapid activation and inactivation of the elimination mechanism is needed to avoid unwanted secondary consequences arising from prolonged interaction with the host's genome. In early studies, plants were co-transformed with selectable markers and genes of interest on separate *Agrobacterium tumefaciens* tumor-inducing DNAs (T-DNAs) and marker-free plants were subsequently isolated through segregation from lines in which the two genes had integrated into unlinked loci[6].

Apart from time considerations, inefficiencies in this approach arise from the difficulty of controlling parameters such as the frequency of co-transformation and the extent to which the two foreign genes are linked. A more recent variation of this approach involves the use of double right-border binary vectors carrying two copies of the T-DNA right border flanking a selectable marker, followed by the gene of interest and one copy of the left-border sequence[13]. Provided that a large number of transformants are screened, it should be possible to separate two types of T-DNA inserts (one containing the selectable marker and gene of interest, and the other containing only the gene) by segregation.

**Table 2. Dominant-negative selectable markers permit assessment of transgene elimination by killing transgene-expressing cells**

| Gene | Gene product | Selection agent | Gene source | Ref. |
|---|---|---|---|---|
| *tms2* | Indoleacetic acid hydrolase | Naphthaleneacetamide | *Agrobacterium tumefaciens* | 14 |
| *codA*[P] | Cytosine deaminase | 5-Fluorocytosine | *Escherichia coli* | 23 |
| *CYP105A*[P] | Cytochrome $P450_{SU1}$ | Sulfonylurea R7402 | *Streptomyces griseolus* | 51 |
| *dhlA* | Bacterial dehalogenase | Dihaloalkanes | *Xanthobacter autotrophicus* | 52 |

[P]Shown to be functional in plastids[24,53].

## Principles of site-specific recombinases used in plants

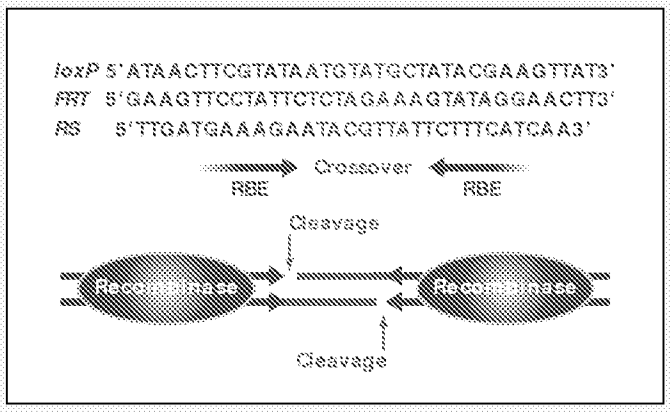


**Figure 1.** Recognition sites for recombinases shown to function in plants share a similar design. All comprise palindromes, which flank the six to eight innermost base pairs. Each recombinase binding element (RBE) is bound by a single recombinase subunit. Cleavage of the sites occurs at the borders between the RBEs and the core sequence. The core element is the site of strand exchange and confers directionality on the recombination site. Recombination requires two recombinase recognition sites bound by four identical recombinase subunits.

The apparatus used by prokaryotes and lower eukaryotes to perform site-specific recombination between specific target sites is well suited to excising selectable markers from plants. All site-specific recombinase systems shown to function in plants[4,6,9] are members of the integrase family and consist of a recombinase (Cre, Flp, or R) and its corresponding recognition sites for recombination (*loxP*, *FRT*, or *RS*, respectively). Crystal structures of the complexes formed between several site-specific recombinases with their DNA targets have revealed conservation within their catalytic regions and fundamental similarities in their modes of action[43]. The Cre recombinase of bacteriophage P1 converts dimeric phage P1 plasmids into their monomeric constituents through recombination between two directly repeated *loxP* sites in the genome of *E. coli*. The yeast Flp and R recombinases enable efficient replication of plasmids bearing the *FRT* and *RS* sites. Inversion of a segment of the plasmid that is flanked by two oppositely oriented recombination sites promotes replication by switching the relative orientations of the replication forks. In contrast to the simple two-component Cre/*loxP*, Flp/*FRT*, and R/*RS* systems, other recombinases require ancillary proteins and often have more complex recognition sites that may confer topological restraints on recombination efficiencies. For example, coliphage λ integrase (Int) mediates recombination between target *E. coli attB* sites and bacteriophage *attP* sites. These are identical in only 15 positions including the 7 bp central overlap, which is the site of DNA exchange. The arms of *attP* have binding sites of Int and accessory proteins such as the host-encoded integration host factor (IHF) required for integration and excision, as well as the phage-encoded excisionase (Xis) required for excision only. Curiously, DNA flanked by *attP* sites was excised at a low frequency in tobacco plants that do not express λ proteins[14].

Other multi-step approaches to dissociate the selectable marker and desired gene have employed transposable elements, intrachromosomal recombination, or site-specific recombination. The non-autonomous *Ds* transposable element in maize can be modified so that virtually any DNA sequence bound by specific DNA repeats can be excised from one location and transposed to other sites in the presence of the *Ac* transposase. Flanking either the selectable marker or the transgene of interest with *Ds* sequences and subsequently introducing *Ac* into the primary transformants confers mobility on the target and thereby dissociates the co-incorporated genes. The selectable marker and transposase can be removed from the gene of interest by segregation. A second transposon-based strategy in which the marker is flanked with *Ds* elements exploits the occasional failure of transposons to reintegrate into the genome following their excision[1]. However, this approach is limited by the low frequency of this phenomenon.

Recently, a much simpler method based on chromosomal recombination between the 352 bp bacteriophage-λ attachment (*attP*) regions (see "Principles of site-specific recombinases used in plants"; Fig.1) was shown to remove selectable markers from tobacco[14]. This two-step regeneration protocol should produce marker-free plants more quickly than do procedures involving re-transformation or cross-pollination, and should also avoid potential problems associated with expression of Flp site– or R site–specific recombinases (discussed below). However, the overall efficiency of this process appears to be low and many deletions in genes of interest occur through illegitimate recombination[14]. Furthermore, the success of this approach in tobacco was not dependent on the expression of the λ proteins Int (integrase) and IHF (integration host factor) normally required for recombination at these sites. Therefore, the mechanistic basis of the phenomenon is not yet understood and it is not yet known how applicable the system may be in other crops.

### Site-specific recombinase-based systems

The ability of microbial site-specific recombinases to cleave DNA at specific sites and ligate it to the cleaved DNA at a second target sequence has enabled their widespread use in manipulating DNA in higher eukaryotes (see "Principles of site-specific recombinases used in plants"). The excision of intervening DNA that accompanies recombination between recognition sites in a direct (head-to-tail) repeat (Fig. 2A) has been exploited to eliminate unwanted transgenic material from the nuclear genome of plants by expression of genes encoding the Cre[10,15–18], Flp[4,11,19], or R[20,21] recombinases. Formally, the product sites generated by excisive recombination are themselves substrates for integrative recombination. However, re-insertion of the elimination cassette is not detected[15,20] for two reasons: first, excision is an intramolecular event, whereas integration requires interaction between unlinked sites; and second, the excised circle cannot replicate autonomously and is probably rapidly lost *in vivo*.

An understanding of other potential consequences of recombinase expression in a heterologous system (Fig. 2B, C) is relevant to their judicious use in plants. Unlike most recombinases, Cre, Flp, and R do not require modification or host-specific factors in order to function in plants. The use of site-specific recombinases may also be a valuable precautionary measure in avoiding a common source of transgene silencing. Transformation protocols for certain species tend to incorporate multiple copies of the same gene at a single locus. These complex integration patterns can be resolved to a single gene copy by flanking the gene of interest with inverted recombination sites. Flanking the cassette containing the selectable marker and recombinase gene with direct repeats ensures simultaneous elimination of the selectable marker[22].

Until recently, recombinase-mediated gene excision in plants was restricted to the elimination of nuclear genes. Introduction of plastid-targeted Cre into the nuclear genome by either

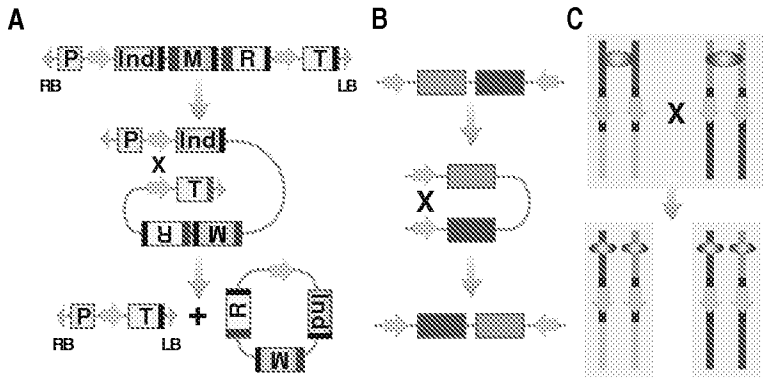


**Figure 2.** Modes of site-specific recombinase action *in vivo*. The nature of DNA rearrangements depends on the relative orientations of the recombinase recognition sites (pink arrows). (A) Recombination between two directly repeated sites excises the intervening DNA as a circular molecule carrying one copy of the recognition site. The second recognition site remains on the targeted molecule. A generic plant transformation cassette for marker elimination[15] is shown. The coding sequence for a chemically inducible transactivator (Ind) and transcriptional units encoding the selectable marker (M) and chemically inducible recombinase (R) are placed between two directly repeated recombinase recognition sites. After elimination of these elements, the presence of only a single recognition site between the transgene of interest (T) and the promoter (P) originally used to express the transactivator results in expression of the transgene of interest. RB, right border; LB, left border of T-DNA. (B) Site-specific recombination between two inverted target sites on a linear molecule inverts the intervening DNA. (C) If two linear molecules carry target sites, recombination leads to exchange of sequences distal to the recombination site[50]. This type of recombination occurs after DNA replication. Top, Replicated chromatids joined by their centromeres before cell division. Bottom, chromosomes of two daughter cells with their normal diploid DNA content after mitosis.

*Agrobacterium*-mediated transformation or pollination can also eliminate selectable markers from the plastome[23,24]. However, the occurrence of frequent DNA deletions in plastid DNA from clones transformed with *Agrobacterium*, and to a lesser extent in those derived from crossing with a Cre-expressing line[23,24], highlights a major limitation of Cre-based systems that has previously been revealed in yeast and mammalian systems. *In vitro* studies indicate that Cre is capable of catalyzing recombination between certain naturally occurring "pseudo-*loxP* sites" sequences that can be highly divergent from the *loxP* consensus sequence[25]. It was subsequently demonstrated that prolonged exposure to Cre caused chromosomal rearrangement in spermatids[26] and that markedly reduced proliferation of Cre-expressing cultured mammalian cells was dependent on Cre activity and associated with numerous chromosomal aberrations[27]. Data regarding the presence of cryptic *FRT* or *RS* sites or the infidelity of Flp- or R-recombinase activities in higher eukaryotes do not appear to be available.

Although there seem to be no reports of chromosomal aberrations in plants where Cre activity is restricted to the nucleus, this might be attributed to a failure to assess genomic stability over several generations of Cre-expressing lines. Indeed, crinkled leaves and reduced fertility have been observed in certain lines expressing high levels of Cre, but these phenotypes do not appear to result from heritable genetic changes[9]. The presence of cryptic *gix* sites in plant genomes might account for the failure to regenerate plants that constitutively express the Gin recombinase from bacteriophage Mu[9].

An obvious approach to restricting the duration of recombinase expression in the plastid to the time required for gene excision is to limit Cre expression by placing it under the control of the tightly regulated XVE-inducible system[28]. As discussed below, regulated Cre expression has been shown to efficiently excise nuclear genes[15]. Alternatively, it may be possible to restrict the toxicity of Cre in plastids by exploiting the efficient RecA-type system of homologous recombination in plastids. In transplastomic tobacco plants, the flanking of a selectable marker with direct repeats enabled its specific elimination using the native plastid homologous recombination apparatus[8,29].

Another approach to prevent excessive exposure to recombinase and simultaneously avoid the long generation times required to introduce and remove stably incorporated recombinase genes is transient exposure of plants to agrobacteria that express the recombinase. Transcription arising from unintegrated T-DNA molecules can be sufficient to eliminate the selectable marker without integration of *Cre* or *Flp* genes into the genome[16,19,30]. Theoretically, the use of two different conditional-lethal dominant markers (Table 2) could facilitate selection of plants that had undergone excision without insertion of the T-DNA bearing the recombinase gene.

Drawbacks of this approach are its extremely low efficiency[16] and also that a significant percentage of the infected lines (around two-thirds in the case of tobacco[16]) that undergo Cre-mediated excision have been transformed with the T-DNA bearing the Cre variant. As it seems that plant host factors are responsible for T-DNA ligation–integration reactions[31], the manipulation of agrobacteria to limit the integration of recombinase genes seems unrealistic. One approach to circumvent this problem might exploit the demonstration that fusions between Cre and the agrobacterium VirE2 and VirF proteins retain recombinase activity and can be transported into plant cells independently of T-DNA transfer[32]. Alternatively, transient Cre expression without T-DNA integration may be feasible in species capable of T-DNA uptake, but with a low susceptibility to T-DNA incorporation. The main limitation of these approaches toward transient exposure to recombinases is that the additional regeneration step required is likely to yield a high frequency of chimeric plants incapable of transmitting the desired trait to their progeny.

## Chemically regulated promoters

The germinal inheritance of site-specific recombinase–mediated deletions is essential in biotechnological applications involving sexually propagated crops. Where this has been investigated for the FLP-*FRT* and R-*RS* systems, most studies have indicated either very limited or no transmission of the recombined state to the next generation[33].

Caution is certainly warranted in distinguishing between the removal of transgenes from somatic cells and DNA recombination in germ-line cells. The identification of even a very high frequency of GM lines in which transgene elimination has occurred may be misleading if only a small fraction of the cells within each line have indeed lost the selectable marker and recombination has not occurred in gametes or their L2 progenitor cells. In addition, when providing FLP recombinase in *trans* and then segregating out, increasing the frequency of transmission of the enzyme to progeny appears to be best facilitated by selecting parent lines that facilitate early recombination before seed maturation[34].

However, chemically regulated marker gene excision strategies that provide the trait gene, the selectable marker, the recombinase gene, and its recognition sites within a single vector have now simplified such multi-step procedures. Prevention of recombinase expression in the absence of its inducer is also likely to limit unin-

tentional damage to native DNA sequences and to ensure that the selectable markers are eliminated only after all transformants have been identified. Flanking the recombinase gene with its own recognition sites ensures that the recombinase will excise the gene directing its own synthesis as soon as the critical level of expression required for excision is reached, thereby avoiding excessive recombinase activity. An unintended basal rate of recombinase gene expression will prevent regeneration of transgenic plants owing to the loss of the marker gene.

Thus far, the efficacy of two variations of this self-deleting system of recombinase expression has been demonstrated in several plant species[15,20,21]. Using the glutathione-*S*-transferase (GST)–MAT (multi-autotransformation) vector system[20], tobacco transformants are selected by "marker-free" screening involving *ipt*-mediated regeneration in the absence of exogenous cytokinin. Excision of both the *R* and the *ipt* genes is regulated by placing *R* recombinase under control of the maize GST-II-27 promoter, which is induced by the herbicide antidote "Safener." Marker-free plants are generated at a frequency of approximately 14%; no data are available on germline transmission. The same approach with slight modification has been subsequently applied to the generation of marker-free transgenic rice, aspen, and snapdragon plants[21].

In the CLX (Cre/*loxP* DNA excision) system shown to function in *Arabidopsis*[15], Cre expression is placed under the control of the β-estradiol-inducible XVE hybrid transactivator. Inclusion of both transcription units required for recombinase activity and antibiotic resistance within *loxP* sites ensures that a negative feedback loop restricts *cre* expression to the level required for excision. β-estradiol-induced excision of the transcriptional units encoding *cre*, the XVE transactivator, and the kanamycin resistance marker activates a downstream green fluorescent protein gene by bringing it into proximity with the G10-90 promoter[15]. An intron within the *cre* gene prevents its expression in *Escherichia coli* during cloning, thereby preventing excision of genetic material contained between the *loxP* sites. Using this system, excision of the elimination cassette occurred in all 19 primary transformants. However, by far the most significant feature of the CLX system is that an estimated 29–66% efficiency is reported for recombination within L2 progenitor cells in the shoot apical meristem of primary transformants[15], with the potential for further improvement of this frequency. This system (represented as a generic transformation cassette in Fig. 2A) therefore ensures inheritance of the excision event in a significant percentage of the progeny of lines exposed to inducer. The CLX system seems to be the most suitable target for future refinement efforts aimed at optimization for use in different crops.

## Fine-tuning contemporary transgene elimination strategies

A limitation encountered when using site-specific recombinases to excise DNA is that every elimination event leaves a residual recognition sequence at the recombination site. Removal of these elements might be prudent in instances where gene "stacking" and several marker elimination steps have resulted in multiple copies of the same recombination site distributed throughout the genome. Residual recombinase recognition sequences may be sites of chromosomal rearrangements upon subsequent exposure to the recombinase. In certain instances (e.g., the generic vector system depicted in Fig.2A) where the recognition sequence may be transcribed, multiple copies of the same element might activate gene silencing mechanisms and thereby counteract the engineering of the desired trait. In cases in which the excision event activates a gene of interest by bringing it into proximity with a promoter[15], one approach to avoid the possibility of gene silencing would be to place the recombination site immediately adjacent to the TATA box of the promoter. As transcription normally begins approximately 30 nucleotides downstream of TATA sequences, transcription of only a few nucleotides at the distal end of the recombinase recognition site would be unlikely to activate gene silencing mechanisms. This approach would not address the possibility that intermolecular or intramolecular recombination between residual sites might generate chromosome deletions, inversions, or translocations.

The most obvious solution to this problem is the sequential use of different recombinases. The recent demonstration that directed evolution strategies can be used to modify recombinase substrate specificities[35–37] extends the potential of this approach. Although functionality of an autoregulatory chemically inducible Flp/*FRT* system has not been demonstrated, it should be functional in crops and may benefit from recent advances in optimizing Flp expression in plants[11,34,38]. Mutant Int proteins no longer require accessory factors from λ phage to perform excisive recombination in human cells[39], but their efficacy in plants remains to be tested. Placing different recombinases under the regulation of a range of chemical-inducible systems currently available[28] will further extend the range and flexibility of different approaches to gene excision. In particular, the use of registered agrochemicals as inducers should expedite the approval of inducible DNA excision cassettes for agricultural use while minimizing the costs of the technology.

Depending on the efficacy of the system in different species, inclusion of a negative selectable marker (Table 2) in the elimination cassette might be desirable in scaled up operations to select for efficient excision. The incorporation of one or more internal ribosome entry site(s)[41] would make the CLX system amenable to activation of multiple transgenes following removal of the elimination cassette.

A more sophisticated approach toward avoiding residual recombination sites is to use tailor-made recombinases[35–37] designed to recognize unique sites within the genome of the crop to be modified. The success of this strategy depends on the availability of methods for efficient gene targeting in higher plants. An additional benefit of combining recombinase-based gene excision strategies with new approaches to ensure the precise integration of foreign DNA is that excisive recombination may itself be sensitive to position effects if target genes lie in a chromatin configuration inaccessible to the recombinase. Currently, the only known way to increase homologous recombination by several orders of magnitude is the induction of genomic double strand breaks[41]. A recent suggestion for achieving gene targeting in plants with the use of both a site-specific recombinase and a site-specific endonuclease[41] could be modified to ensure the simultaneous elimination of "used" components, leaving only a residual recombinase recognition site with the gene of interest. *Fok*I–zinc finger chimeric nucleases[42] and group II introns modified to insert into specific loci[44] might also be useful in creating the next generation of transformation vectors capable of both gene targeting and the subsequent elimination of unnecessary foreign DNA.

Although such proposals remain highly speculative, it is worth considering that crop improvement in the post-genomic era will most likely be limited more by the availability of suitable transformation technologies than by the rate of gene discovery. Irrespective of whether or not transformation efficiencies can be enhanced to a level at which the use of selectable markers becomes unnecessary, refinements in the technology to precisely excise foreign genetic material will continue to form an integral part of strategies to improve transformation rates and to safeguard against the unintended spread of genes encoding novel traits.

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1. Ebinuma, H., Sugita, K., Matsunaga, E. & Yamakado, M. Selection of marker-free transgenic plants using the isopentenyltransferase gene. *Proc. Natl. Acad. Sci. USA* **94**, 2117–2121 (1997).
2. Kunkel, T., Niu, Q.-W., Chan, Y.-S. & Chua, N.-H. Inducible isopentenyltransferase as a high-efficiency marker for plant transformation. *Nat. Biotechnol.* **17**, 916–919 (1999).
3. Zuo, J., Niu, Q.-W., Ikeda, Y. & Chua, N.-H. Marker-free transformation: increasing transformation frequency by the use of regeneration-promoting genes. *Curr. Opin. Biotechnol.*, in press (2002).
4. Hohn, B., Levy, A.A. & Puchta, H. Elimination of selection markers from transgenic plants. *Curr. Opin. Biotechnol.* **12**, 139–143 (2001).
5. Ow, D. Marker genes. in *Joint FAO/WHO Expert Consultation on Foods Derived from Biotechnology*, World Health Organization, May 29–June 2, 2000 (World Health Organization, Geneva, Switzerland, 2000). http://www.who.int/fsf/GMfood/Consultation_May2000/Biotech_00_14.pdf.
6. Ebinuma, H. *et al.* Systems for the removal of a selection marker and their combination with a positive marker. *Plant Cell Rep.* **20**, 383–392 (2001).
7. Daniell, H., Khan, M.S. & Allison, L. Milestones in chloroplast genetic engineering: an environmentally friendly era in biotechnology. *Trends Plant Sci.* **7**, 84–91 (2002).
8. Maliga, P. Engineering the plastid genome of higher plants. *Curr. Opin. Plant Biol.* **5**, 164–172 (2002).
9. Ow, D.W. Recombinase-directed plant transformation for the post genomic era. *Plant Mol. Biol.* **48**, 183–200 (2002).
10. Hoff, T., Schnorr, K.M. & Mundy, J. A recombinase-mediated transcriptional induction system in transgenic plants. *Plant Mol. Biol.* **45**, 41–49 (2001).
11. Luo, H., Lyznik, L.A., Gidoni, D. & Hodges, T.K. FLP-mediated recombination for use in hybrid plant production. *Plant J.* **23**, 423–430 (2000).
12. Keenan, R.J. & Stemmer, W.P.C. Nontransgenic crops from transgenic plants. *Nat. Biotechnol.* **20**, 215–216 (2002).
13. Lu, H.-J., Zhou, X.R., Gong, Z.-X. & Upadhyaya, N.M. Generation of selectable marker-free transgenic rice using double right-border (DRB) binary vectors. *Aust. J. Plant Physiol.* **28**, 241–248 (2001).
14. Zubko, E., Scutt, C. & Meyer, P. Intrachromosomal recombination between attP regions as a tool to remove selectable marker genes from tobacco transgenes. *Nat. Biotechnol.* **18**, 442–445 (2000).
15. Zuo, J., Niu, Q.-W., Møller, S.G. & Chua, N.-H. Chemical-regulated, site-specific DNA excision in transgenic plants. *Nat. Biotechnol.* **19**, 157–161 (2001).
16. Gleave, A.P., Mitra, D.S., Mudge, S.R. & Morris, B.A.M. Selectable marker-free transgenic plants without sexual crossing: transient expression of cre recombinase and use of a conditional lethal dominant gene. *Plant Mol. Biol.* **40**, 223–235 (1999).
17. Dale, E.C. & Ow, D.W. Gene transfer with the subsequent removal of the selection gene from the host genome. *Proc. Natl. Acad. Sci. USA* **88**, 10558–10562 (1991).
18. Russell, S.H., Hoopes, J.L. & Odell, J.T. Directed excision of a transgene from the plant genome. *Mol. Gen. Genet.* **234**, 49–59 (1992).
19. Lyznik, L.A., Rao, K.V. & Hodges, T.K. FLP-mediated recombination of FRT sites in the maize genome. *Nucleic Acids Res.* **24**, 3784–3789 (1996).
20. Sugita, K., Kasahara, T., Matsunaga, E. & Ebinuma, H. A transformation vector for the production of marker-free transgenic plants containing a single copy transgene at high frequency. *Plant J.* **22**, 461–469 (2000).
21. Ebinuma, H. & Komamine, A. MAT (Multi-Auto-Transformation) Vector System. The oncogenes of *Agrobacterium* as positive markers for regeneration and selection of marker-free transgenic plants. *In Vitro Cell. Dev. Biol. Plant* **37**, 103–113 (2001).
22. Srivastava, V., Anderson, O.A. & Ow, D.W. Single-copy transgenic wheat generated through the resolution of complex integration patterns. *Proc. Natl. Acad. Sci. USA* **96**, 11117–11121 (1999).
23. Corneille, S., Lutz, K., Svab, Z. & Maliga, P. Efficient elimination of selectable marker genes from the plastid genome by the CRE–*lox* site-specific recombination system. *Plant J.* **27**, 171–178 (2001).
24. Hajdukiewicz, P.T.J., Gilbertson, L. & Staub, J.M. Multiple pathways for Cre/*lox*-mediated recombination in plastids. *Plant J.* **27**, 161–170 (2001).
25. Thyagarajan, G., Guimaraes, M.J., Groth, A.C. & Calos, M.P. Mammalian genomes contain active recombinase recognition sites. *Gene* **244**, 47–54 (2000).
26. Schmidt, E.E., Taylor, D.S., Prigge, J.R., Barnett, S. & Capecchi, M. Illegitimate Cre-dependent chromosome rearrangements in transgenic mouse spermatids. *Proc. Natl. Acad. Sci. USA* **97**, 13702–13707 (2000).
27. Loonstra, A. *et al.* Growth inhibition and DNA damage induced by Cre recombinase in mammalian cells. *Proc. Natl. Acad. Sci. USA* **98**, 9209–9214 (2001).
28. Zuo, J. & Chua, N.-H. Chemical-inducible systems for regulated expression of plant genes. *Curr. Opin. Biotechnol.* **11**, 146–151 (2000).
29. Iamthan, S. & Day, A. removal of antibiotic resistance genes from transgenic tobacco plastids. *Nat. Biotechnol.* **18**, 1172–1176 (2000).
30. Srivastava, V. & Ow, D.W. Single-copy primary transformamts of maize obtained through the co-introduction of a recombinase-expressing construct. *Plant Mol. Biol.* **46**, 561–566 (2001).
31. Ziemienowicz, A., Tinland, B., Bryant, J., Gloeckler, V. & Hohn, B. Plant enzymes but not *Agrobacterium* VirD2 mediate T-DNA ligation *in vitro*. *Mol. Cell. Biol.* **20**, 6317–6322 (2000).
32. Vergunst, A.C. *et al.* VirB/D4-dependent protein translocation from *Agrobacterium* into plant cells. *Science* **290**, 979–982 (2000).
33. Davies, G.J., Kilby, N.J., Riou-Khamlichi, C. & Murray, J.A.H. Somatic and germinal inheritance of an FLP-mediated deletion in transgenic tobacco. *J. Exp. Bot.* **50**, 1447–1456 (1999).
34. Gidoni, D. *et al.* Embryonal recombination and germline inheritance of recombined *FRT* loci mediated by constitutively expressed FLP in tobacco. *Euphytica* **121**, 145–156 (2001).
35. Buchholz, F. & Stewart, A.F. Alteration of Cre recombinase site specificity by substrate-linked protein evolution. *Nat. Biotechnol.* **19**, 1047–1052 (2001).
36. Sclimenti, C.R., Thayagarajan, B. & Calos, M.P. Directed evolution of a recombinase for improved genomic integration at a native human sequence. *Nucl. Acids Res.* **29**, 5044–5051.
37. Santoro, S.W. & Schultz, P.G. Directed evolution of the site specificity of Cre recombinase. *Proc. Natl. Acad. Sci. USA* **99**, 4185–4190 (2002).
38. Baszczynski, C.L. *et al.* Novel nucleic acid sequence encoding FLP recombinase. US patent 6,175,058 (2001).
39. Lorbach, E., Christ, N., Schwikardi, M. & Dröge, P. Site-specific recombination in human cells catalyzed by phage λ integrase mutants. *J. Mol. Biol.* **296**, 1175–1181 (2000).
40. Urwin, P., Li, Y., Martin, H., Atkinson, H. & Gilmartin, P.M. Functional characterization of the EMCV IRES in plants. *Plant J.* **24**, 583–589 (2000).
41. Kumar, S. & Fladung, M. Controlling transgene integration in plants. *Trends Plant Sci.* **6**, 155–159 (2001).
42. Bibikova, M. *et al.* Stimulation of homologous recombination through targeted cleavage by chimeric nucleases. *Mol. Cell. Biol.* **21**, 289–297 (2001).
43. van Duyne, G.D. A structural view of Cre-*loxP* site-specific recombination. *Annu. Rev. Biophys. Biomol. Struct.* **30**, 87–104 (2001).
44. Guo, H. *et al.* Group II introns designed to insert into therapeutically relevant DNA target sites in human cells. *Science* **289**, 452–457 (2000).
45. Kuiper, H.A., Kleter, G.A., Noteborn, H.P.J.M. & Kok, E.J. Assessment of the food safety issues related to genetically modified foods. *Plant J.* **27**, 503–528 (2001).
46. Goddijn, O.J.M., Schouten, P.M.V., Schilperoort, R.A. & Hoge, J.H.C. A chimeric tryptophan decarboxylase gene as a novel selectable marker in plant cells. *Plant Mol. Biol.* **22**, 907–912 (1993).
47. Joersbo, M. & Okkels, F.T. A novel principle for selection of transgenic plant cells: positive selection. *Plant Cell Rep.* **16**, 219–221 (1996).
48. Haldrup, A., Petersen, S.G. & Okkels, F.T. The xylose isomerase gene from *Thermoanaerobacterium thermosulfurogenes* allows effective selection of transgenic plant cells using D-xylose as the selection agent. *Plant Mol. Biol.* **37**, 287–296 (1998).
49. Joersbo, M. *et al.* Analysis of mannose selection used for transformation of sugar beet. *Mol. Breed.* **4**, 111–117 (1998).
50. Qin, M., Bayley, C., Stockton, T. & Ow, D.W. Cre recombinase-mediated site-specific recombination between plant chromosomes. *Proc. Natl. Acad. Sci. USA* **91**, 1706–1710 (1994).
51. O'Keefe, D.P. *et al.* Plant expression of a bacterial cytochrome P450 that catalyzes activation of a sulfonylurea pro-herbicide. *Plant Physiol.* **105**, 473–482 (1994)
52. Næstaed, H. *et al.* A bacterial haloalkane dehalogenase gene as a negative selectable marker in *Arabidopsis*. *Plant J.* **18**, 571–576 (1999).